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Loss of Interleukin 6 Results in Delayed Mammary Gland Involution: A Possible Role for Mitogen-Activated Protein Kinase and Not Signal Transducer and Activator of Transcription 3

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We have investigated the role of IL-6 in the initiation and progression of mouse mammary gland involution in IL-6-null mice. This study was based on the hypothesis that IL-6 is the activating cytokine for signal transducer and activator of transcription 3 (Stat), the transcription factor whose presence is required for controlled mammary gland involution. We now show that expression of IL-6 is low during lactation but increases at the onset of involution in parallel with the activation of Stat3 and p44/42 MAPK. Moreover, we demonstrated that injection of IL-6 into virgin and lactating mice activates Stat3 in mammary epithelium. The *in vivo* role of IL-6 was investigated using mutant mice. Involution of mammary tissue in IL-6-null mice was delayed similar to that seen in mammary conditional Stat3- and Bax-null mice. However, Stat3 activation during involution was independent of the IL-6 status. This suggests that either IL-6 does not induce Stat3 *in vivo* or its absence is compensated for by other cytokines, such as leukemia-inhibitory factor (LIF). In contrast, the increase of p44/42 MAPK (ERK1/2) phosphorylation at the onset of involution was dependent on the presence of IL-6. Delayed involution corresponded with a decrease of epithelial cell death, and a delayed induction of Bax and sulfated glycoprotein 2 (SGP2, or clusterin) expression. Our experiments demonstrate on a genetic level that IL-6 contributes to the induction of the controlled remodeling of mammary tissue during involution, possibly through the MAPK pathway and by mediating the expression of the cell death protein Bax. (*Molecular Endocrinology* 16: 2902–2912, 2002)

MEMBERS OF THE IL-6 cytokine family contribute to a variety of biological processes, including immune response, inflammation, hematopoiesis, and oncogenesis by regulating cell growth, survival, and differentiation (1). These cytokines use gp130 as a common receptor subunit, and the binding of ligands activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction pathway and also, to a lesser extent, the MAPK and phosphatidylinositol 3-kinase (PI3K) pathways. Stat3 plays a central role in transmitting signals from the membrane to the nucleus, and it is essential for gp130-mediated cell survival and G1-to-S cell cycle-transition signals (2). Stat3 is also required for gp130-mediated maintenance of the pluripotent state of proliferating embryonic stem cells (through LIF) and for gp130-induced macrophage differentiation (2). Furthermore, Stat3 regulates cell movement, such as leukocyte, epidermal cell, and keratinocyte migration, and it regulates B cell differentiation into antibody-forming plasma cells (1).

STAT proteins transduce signals from the cell membrane to the nucleus where they activate transcription of specific target genes. Stat3 is activated through phosphorylation on tyrosine 705 in response to many cytokines, including the epidermal growth factor (EGF) and IL-6 (3). In mammary tissue, Stat3 is preferentially phosphorylated during the estrous cycle, at midpregnancy (4), and during the first stage of involution (5). Inactivation of the Stat3 gene in mammary epithelium of mice results in a delayed involution (6, 7), suggesting a controlling role of Stat3. Although the mechanism of Stat3 activation in mammary epithelium during involution is not known, increased milk pressure could be an initial inducer. Studies in the rat heart demonstrated that Stat3 and gp130 are phosphorylated and thereby activated upon an increase of pressure. In parallel, the expression levels of IL-6 and cardiotropin 1 (CT-1) increased in the heart (8). Similarly, mechanical stretch on cardiomyocytes activates gp130 and Stat3 (9), which supports the notion that overload pressure or the direct mechanical stretch might be inducers of the IL-6 production and Stat3 phosphorylation. Mammary gland involution can be induced by interrupting breast-feeding in the human, and also by

Abbreviations: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IL-6-R, IL-6 receptor; JAK, Janus kinase; JNK, Jun N-terminal kinase; L10, d 10 of lactation; LIF, leukemia-inhibitory factor; O/N, overnight; PI3K, phosphatidylinositol 3-kinase; RT, room temperature; SAPK, stress-activated protein kinase; Stat, signal transducer and activator of transcription; WAP, whey acidic protein.

removing pups from lactating mice. In both cases, the accumulation of milk produced by the mammary gland will increase the intramammary pressure and thus cause a mechanical stretch to the mammary epithelium.

IL-6 has diverse functions, such as promoting proliferation (10) and apoptosis (11, 12), possibly by using different signaling pathways. IL-6 was detected in bovine mammary epithelium (13), normal human breast epithelial cells (14), and adipocytes (15), and it inhibits the proliferation of T47D breast cancer cells *in vitro* (11). Thus, we hypothesized that IL-6 could be an inducer of involution, possibly through the activation of Stat3. We performed a mouse expression sequence tag database search and determined that IL-6 is highly expressed in both mammary tumors and normal lactating glands, further suggesting that this cytokine plays a role in mammary development and function. To address the question whether IL-6 controls mammary gland involution, we have now used a genetic approach and investigated the ability of mammary tissue to function and undergo involution in the absence of IL-6.

RESULTS

Activation of Stat3 in Involuting Mammary Tissue

To identify the cytokine(s) that induces cell death in the mammary tissue, we employed the well-defined nipple-sealing experimental model (5). In this model two nipples from a d 10 lactating mouse are sealed, which results in epithelial cell death within the corresponding glands despite the presence of circulating lactogenic hormones (5). In contrast, the open glands continue to be suckled and their epithelia do not show signs of significant apoptosis.

Mammary tissue was isolated from open and sealed glands at 12, 24, 48, and 72 h after the initial sealing event, and the phosphorylation status of Stat3 was analyzed (Fig. 1). Phosphorylation on both tyrosine and serine residues was detected preferentially in mammary tissue from the sealed glands. The levels of total Stat3 were similar in mammary tissue from open and sealed glands. Immunohistochemistry established nuclear localization of Stat3 in the sealed (Fig. 2A) but not in the open (Fig. 2B) glands at 48 h. On the other hand, nuclear localization of Stat5a was observed in epithelium from open and sealed glands (Fig. 2, C and D). These results are consistent with previous studies (5), and they suggest that the nipple-sealing model is appropriate for the identification of factors/cytokines that may control early stages of involution. The presence of gp130, the common receptor subunit for IL-6 family cytokines, in mammary tissue from open and sealed glands was verified by immunoprecipitation studies (Fig. 1). Whereas the protein levels of gp130 were relatively stable in open glands, the amount of gp130 in the sealed glands was highest at 12 h and decreased subsequently. Although the mechanism of this reduction is not known, a negative feedback can be postulated.

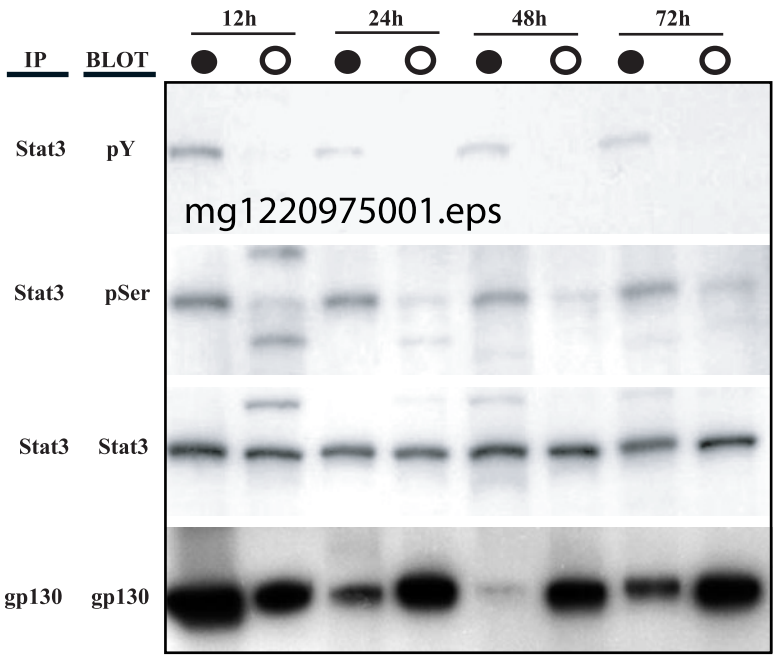

Fig. 1. Immunoprecipitation of Stat3 and gp130 from Sealed and Open Glands at 12, 24, 48, and 72 h

Stat3 is activated (Tyr 705 and Ser 727) during involution of the mammary gland. gp130 Appears in all tissue examined, with the highest at 12 h sealed gland, and it declines at 24, 48, and 72 h sealed glands. On the other hand, the level of gp130 protein in the open gland is stable. ○, Open glands; ●, sealed glands; pY, phosphotyrosine 705-Stat3; pSer, phosphoserine 727-Stat3.

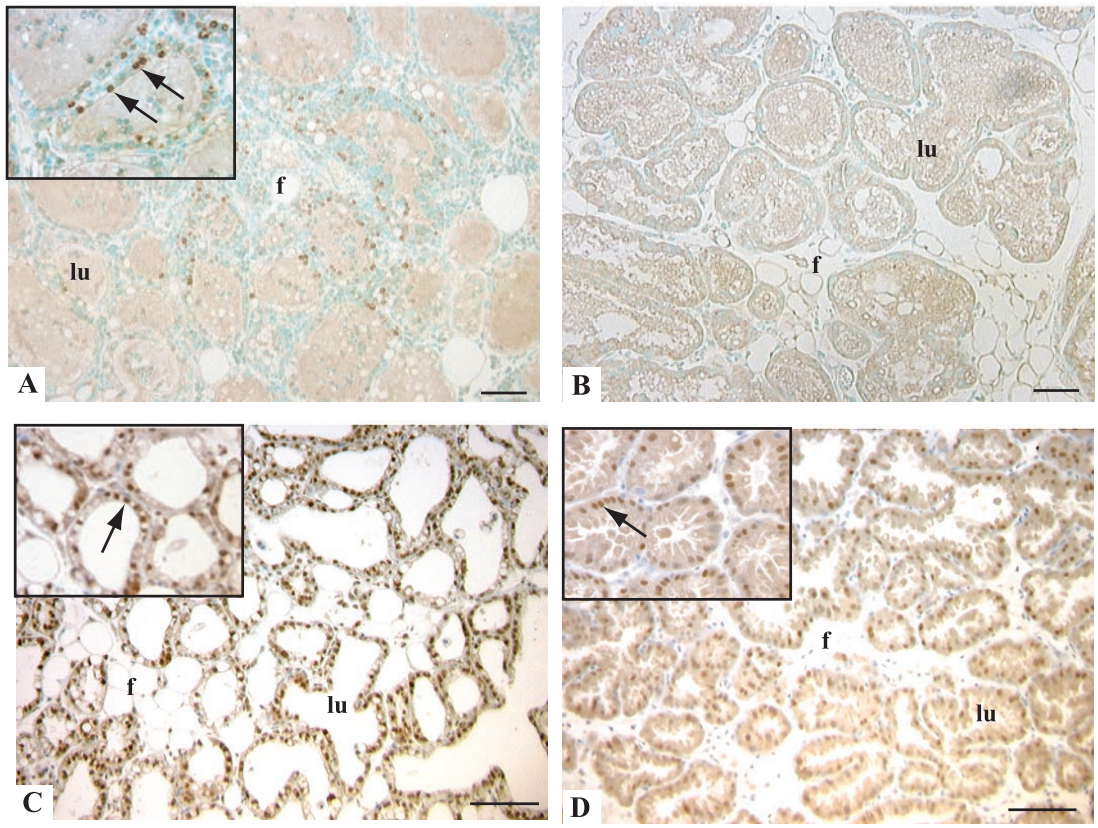

Fig. 2. Immunohistochemistry of Stat3 (A and B) and Stat5a (C and D) in 48-h Sealed (A and C) and Open (B and D) Glands

Stat3 nuclear translocation is detected in sealed (as shown by *arrowheads* in A) but not open (B) glands. Stat5a nuclear localization (as shown by *arrowheads*) is detected in both sealed (C) and open (D) glands. Enlarged pictures are shown on the *left top corner* of the image. Lu, Lumen; f, fat. *Bar*, 100 μm.

IL-6 Is Expressed in Mammary Tissue and Activates Stat3

Expression sequence tag database searches were used to identify the expression of IL-6 and other cytokines in mouse mammary tissue (Table 1). IL-6 transcripts were found in cDNA libraries obtained from mammary tissues of wild-type virgin and lactating mice, and in libraries generated from transgenic mouse mammary tumor models. RT-PCR analyses (Fig. 3) confirmed the results from the database searches. IL-6 mRNA was detected at highest level in sealed glands at 24 h and at reduced levels at 12 and 48 h (Fig. 3). Expression in open glands was much lower. In contrast, gp130 and IL-6 receptor (IL-6-R) mRNA levels were low at 12 h but increased around 24 h at similar levels in open and closed glands (Fig. 3). When we increased PCR cycles for gp130 and IL-6-R, strong bands could be seen in all samples (data not shown). This suggests that gp130 and IL-6-R are present in mammary gland during lactation and involution. In the open and sealed glands, the gp130 mRNA levels appeared in a pattern (Fig. 3) different from its protein level (Fig. 1), suggesting a posttranscriptional control mechanism.

Table 1. Mouse Expression Sequence Tag BLAST Searches of Cytokines and their Receptors in Mouse Mammary Gland and Mammary Tumor Libraries

Cytokines	NCI-CGAP-Mam1–6	NMLMG	NbMMG	RIKEN
gp130	2		1	1
IL-6	10	3	1	
LIF	1			
LIF-R	1	2		
EGF-R	2	1	2	
PDGF-Rβ	2	1	3	
OSM-Rβ	9			
Leptin-R	Placenta, embryo tissues			
IL-10	T cells			
CNTF-R	Brain tissues			

The *numbers* represent the time of high-score hits found in different libraries. LIF-R, LIF receptor; EGF-R, epidermal growth factor receptor; PDGF-Rβ, platelet-derived growth factor receptor β; OSM-Rβ, Oncostatin M receptor β; CNTF-R, ciliary neurotrophic factor receptor.

[a] NCI-CGAP-Mam1–6, mouse mammary tumor libraries; NMLMG, lactating mouse mammary gland library; NbMMG, 4-wk-old male mice mammary gland library. RIKEN, full-length, lactating mouse mammary gland library.

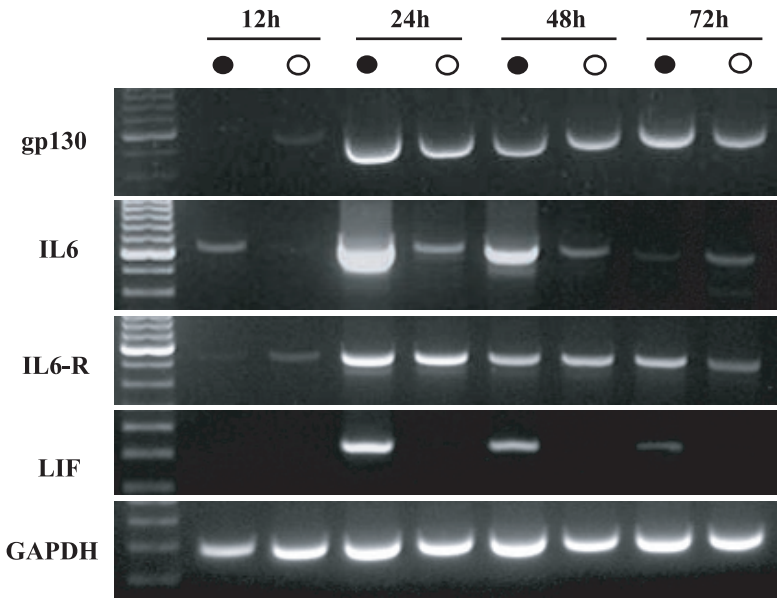

Fig. 3. RT-PCR of gp130, IL-6, IL-6-R, LIF, and GAPDH from Sealed (●) and Open (○) Mammary Glands at Different Time Points

gp 130 and IL-6-R are expressed in all tissues examined. IL-6 appears in sealed gland at 12 h and peaks at 24 h, and then decreases at 48 and 72 h in sealed glands. In the open glands, IL-6 is detected at a low level from 24 h to 72 h. LIF appears in sealed gland at 24, 48, and 72 h and is not detected in the open glands. GAPDH serves as a loading control.

In addition, LIF, another IL-6 family member, was detected at 24 h, 48 h, and 72 h in sealed glands. This suggests that the induction of mammary involution is possibly regulated by multiple cytokines.

We have investigated whether IL-6 can activate Stat3 in the mammary tissue *in vivo*. IL-6 was injected into diestrous virgin and d 10 lactating (L10) mice. Stat3 activation, as evidenced by its nuclear localization and immunoprecipitation (Fig. 4), was analyzed 15 min after injection. Extensive nuclear localization (Fig. 4a) was observed in mammary epithelium. Stat3 tyrosine phosphorylation was detected after IL-6 treatment but not in its absence in virgin (data not shown) and lactating mice (Fig. 4b). No MAPK activity differences were detected in these tissues, which is likely because of the timing of tissue collection.

Involution Is Delayed in IL-6-Null Mammary Tissue

IL-6-null mice are fertile and can fully support their litters. Histological analysis at d 10 of lactation established that the entire fat pad was filled with secretory epithelium and confirmed that the gland had developed and acquired a functional appearance (Fig. 5B). To investigate whether IL-6-null mammary tissue was able to remodel during involution, pups were removed at d 10 of lactation, followed by a histological analysis at d 2, 3, and 4 of involution. At d 2, the histological appearance of wild-type and IL-6-null mammary tissue was similar (data not shown). However, more TUNEL (terminal deoxynucleotidyltransferase-mediated UTP end labeling)-positive cells ($P < 0.01$) were detected in wild-type (4.7 $\pm$ 0.5%, Fig. 6, A and E) than in IL-6-null tissue (2.4 $\pm$ 0.3%, Fig. 6, B and E). At d 3 of involution, the morphology of IL-6-null mammary tissue (Fig. 5D) was distinctly different from control tissue (Fig. 5C). While IL-6-null alveoli appeared to be intact and exhibited a secretory phenotype (Fig. 5F), wild-type alveoli had lost their integrity and contained accumulated apoptotic cells in their lumina (Fig. 5E). Nevertheless, the rate of apoptosis had increased in the IL-6-null tissue (5.7 $\pm$ 0.7%, Fig. 6, D and E) to a level comparable to that seen in the wild-type tissue (6.8 $\pm$ 0.7%, Fig. 6, C and E). At d 4 of involution, the morphology in IL-6-null mice was similar to that seen in wild-type mice (data not shown). These results suggest that IL-6 is an important signal for the initiation of mammary gland involution.

IL-6 signals through several pathways, including JAK/Stat, MAPK, and PI3K, and all three were investigated (Fig. 7). Stat3 phosphorylation (Tyr 705) was observed in mammary tissue from IL-6-null mice during involution in a pattern similar to that seen in wild-type mice. No apparent deregulation was observed for Stat1 and gp130. Actin and E-cadherin served as loading controls. In contrast, Stat5a level remained higher in IL-6-null mice at d 1 of involution compared with control mice, which could be due to a direct effect of IL-6 or the delayed involution. SGP2 and Bax are indicators of involution, and their levels increased at d 1 of involution in wild-type mice (Fig. 7). In contrast, their induction in IL-6-null mice was delayed, which is consistent with our morphological observations (Fig. 5).

We also examined p44/42 (ERK1/2), p38, stress-activated protein kinase (SAPK)/Jun N-terminal kinase

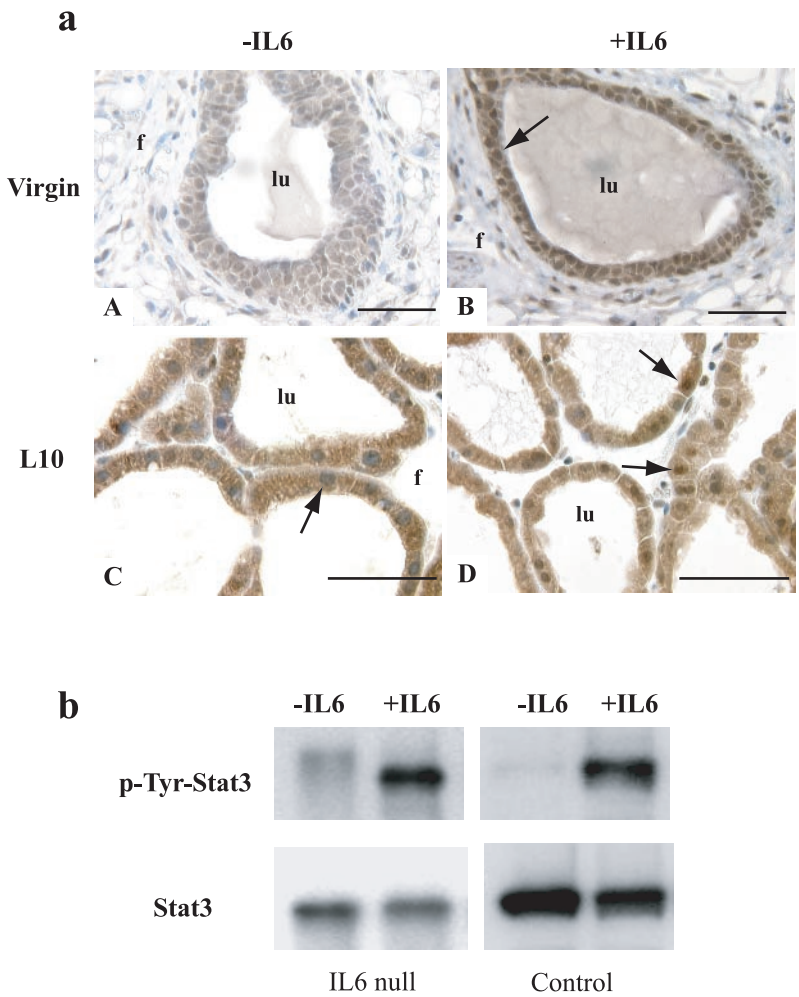

Fig. 4. Injection of IL-6 into Virgin and Lactating Mice Activates Stat3 in Mammary Epithelium

a, Immunohistochemistry of Stat3 in mammary tissues of virgin (A, B) and L10 (C and D) mice before (A and C) and after (B and D) IL-6 injection (ip). Before injection, mammary epithelia from virgin mice (A) show a basal level of Stat3 staining, while the nuclei of epithelia from the L10 mouse (C) are clearly *blue* (*arrowhead*). Stat3 nuclear translocation appears (*arrows*) after IL-6 injection in both virgin (B) and L10 mammary glands (D). lu, Lumen; f, fat. *Bar*, 50 μm. b, Immunoprecipitation of Stat3. Phosphorylated Stat3 (Tyr 705) is detected in the IL-6 treated (+IL6) glands but not in the untreated glands (−IL6).

(JNK) MAPK, and Akt phosphorylation. p44/42 MAPK activation was augmented at d 1 of involution in wild-type mice but to a less extent in IL-6-null mice (Fig. 7). This indicates that the activation of p44/42 MAPK is IL-6 dependent and may be the initial pathway for IL-6 signaling at early involution. On the other hand, p38 and SAPK/JNK MAPK phosphorylation increase, and Akt phosphorylation decreases in IL-6-null mice at d 1 of involution. These are similar to that seen in wild-type mice (data not shown). This suggested that only part of MAPK but no PI3K pathways are important at this stage. The milk protein WAP (whey acidic protein) was detected in all tissues examined, and no apparent difference was observed, which could be due to the milk stasis in the mammary gland at early involution.

Activation of Stat3 and Stat5a, as evidenced by nuclear translocation, was examined at L10 and d 1–3 of involution. Nuclear Stat3 was not detected at L10 (Fig. 8B) but at d 1 of involution with an increase at d 2 and d 3 (Fig. 8, D and F). The activity pattern in IL-6-null mice was similar to that of control mice. Nuclear Stat5a was detected at L10 (Fig. 8A) and throughout involution (Fig. 8, C and E). Since phosphorylation studies (4) demonstrated a sharp decline of phosphorylated Stat5a during the first few days of involution, it is likely that the nuclear Stat5 represents an unphosphorylated form.

DISCUSSION

Involution and controlled remodeling of mouse mammary tissue depends on local and systemic cues. The transcription factor Stat3 is activated in mammary tissue upon weaning (4), and the inactivation of Stat3 in alveolar epithelium of mice results in delayed tissue remodeling during involution (6, 7). Stat3 is the down-

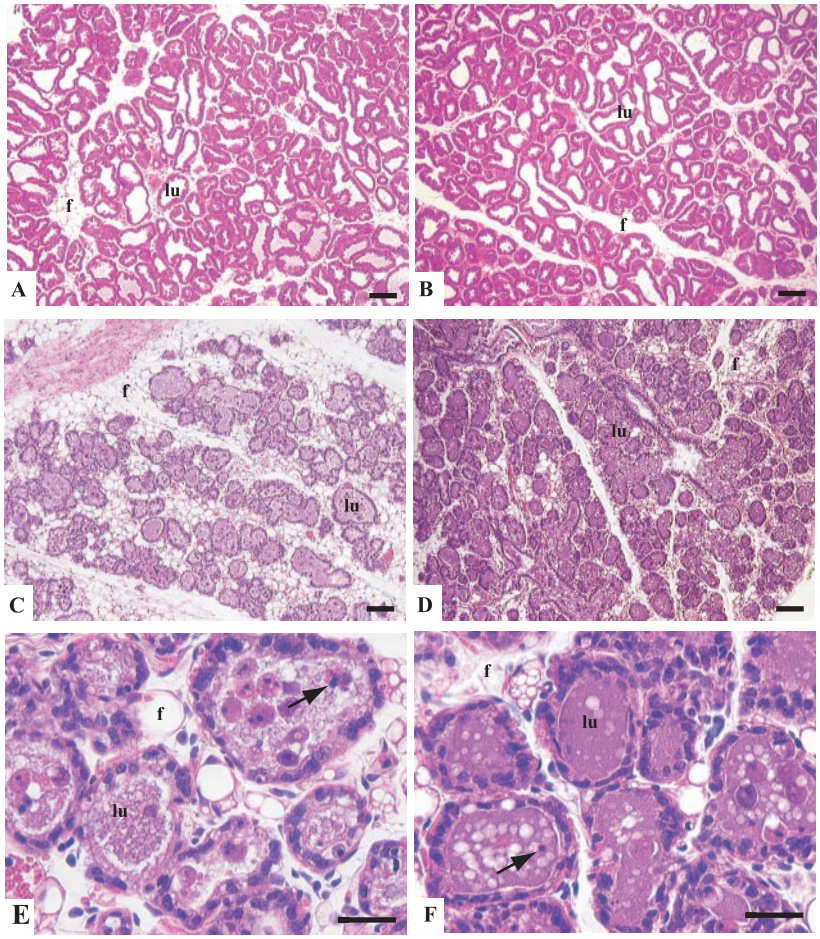

Fig. 5. Histology of Mammary Glands of L10 (A and B) and d 3 Involution (C–F) from Wild-Type (A, C, and E) and IL-6-Null (B, D, and F) Mice (Hematoxylin and Eosin Stain)

There is no obvious histological difference between lactation glands from wild-type (A) and IL-6-null (B) mice. On d 3 of involution, there is less fat tissue (D) and alveoli are more intact in the IL-6-null mice (F) compared with that of wild-type (C and E) mice. Lu, Lumen; f, fat. *Bar,* 200 μm (A–D) and 50 μm (E and F). *Arrows* indicate dying cells.

stream mediator of many cytokines, including the IL-6 family that encompasses IL-6 and LIF (2). However, the activating ligands in mammary epithelium during involution are not known. We hypothesized that IL-6 is the activating cytokine, and we have now demonstrated that the injection of IL-6 can activate Stat3 in mammary tissue. Furthermore, IL-6 expression is induced in mammary tissue at early stages of involution and IL-6-null mice displayed a delayed involution upon the removal of pups. This suggests that IL-6 is an inducer of Stat3-mediated mammary remodeling during involution. However, Stat3 activity was not reduced during involution in IL-6-null mice. We offer two explanations. First, it is possible that IL-6 is not an *in vivo* inducer of Stat3 activity in mammary epithelium. Alternatively, Stat3 activity in IL-6-null mice could be the result of the compensating presence of other cytokines, such as LIF. Notably, LIF can induce Stat3 phosphorylation in mammary tissue, and delayed involution was observed in the absence of LIF (Zhao, L., and L. Hennighausen, unpublished).

Even though there is no direct evidence that IL-6 is an *in vivo* inducer of Stat3 signaling, the genetic evidence clearly points to a role of IL-6 in the onset of involution. IL-6 family cytokines activate not only the Jak/Stat pathway through binding to the gp130 receptor, but also the MAPK pathway (16). The p44/42 MAPK (also known as ERK1/2) cascade represents a ubiquitous cell signaling pathway that plays important roles in a variety of processes, such as cell proliferation, differentiation, and apoptosis (17–19). A rapid increase of phosphorylated p44/42 MAPK was observed at 24 h of involution in wild-type but not IL-6-null mammary tissue, suggesting that p44/42 MAPK plays a role at early stages, either directly through IL-6 or indirectly. Notably, IL-6 expression increased in the first phase of involution within 1 d after nipple sealing, a time when milk has accumulated in the lobules and the alveoli are expanded. How do the loss of IL-6 and the lack of p44/42 MAPK induction affect the physiology of mammary tissue? Mammary development and

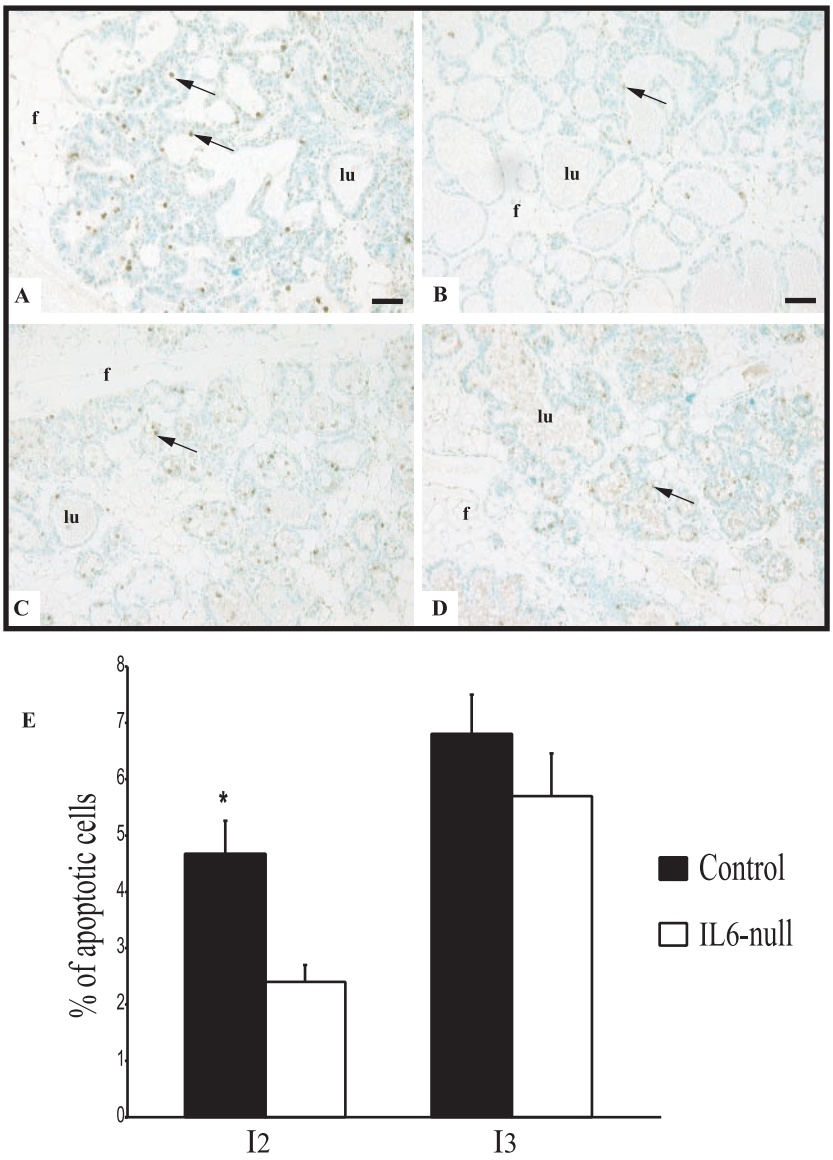

Fig. 6. Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Assay of d 2 (I2, A and B) and day 3 (I3, C and D) Involuting Mammary Glands from Wild-Type (A and C) and IL-6-Null (B and D) Mice

At d 2 of involution, there are more labeled cells (as indicated by *arrows*) in wild-type (A and E) than that in IL-6-null (B and E) mice. However, at d 3 of involution (C, D, and E), the difference is not significant. lu, Lumen; f, fat. *, $P < 0.001$. *Black bar*, control mice; *white bar*, IL-6-null mice. *Bar*, 100 μm (A–D).

function are dependent on the transcription factor Stat5, which is activated by prolactin (20, 21). Levels of Stat5a decrease upon the onset of involution (4), and the delayed decrease during the first few days of involution in IL-6-null mice suggests the functional maintenance of mammary epithelium. How can it be explained that the induction of the cell proliferation/survival MAPK pathway may control mammary epithelial cell death during involution? Differentiated mammary epithelial cells are arrested in G0, and for these cells to undergo apoptosis they need to enter the cell cycle (22, 23). Induction of MAPK could accomplish this (24), and the loss of normal activation of this pathway in the absence of IL-6 could result in the retention of epithelial cells in G0. However, the absence of IL-6 results only in a limited delay of involution, and other compensating signals eventually initiate involution.

The potential importance of the IL-6-gp130-MAPK axis extends beyond the remodeling process of mammary epithelium. The number of thymocytes and peripheral T cells are greatly reduced in IL-6-null mice (25), which may be the result of defective Stat3 or MAPK signaling. The function of IL-6 is clearly cell specific, as IL-6 induces proliferation of a B cell hybridoma cell line (10) but inhibits the proliferation of

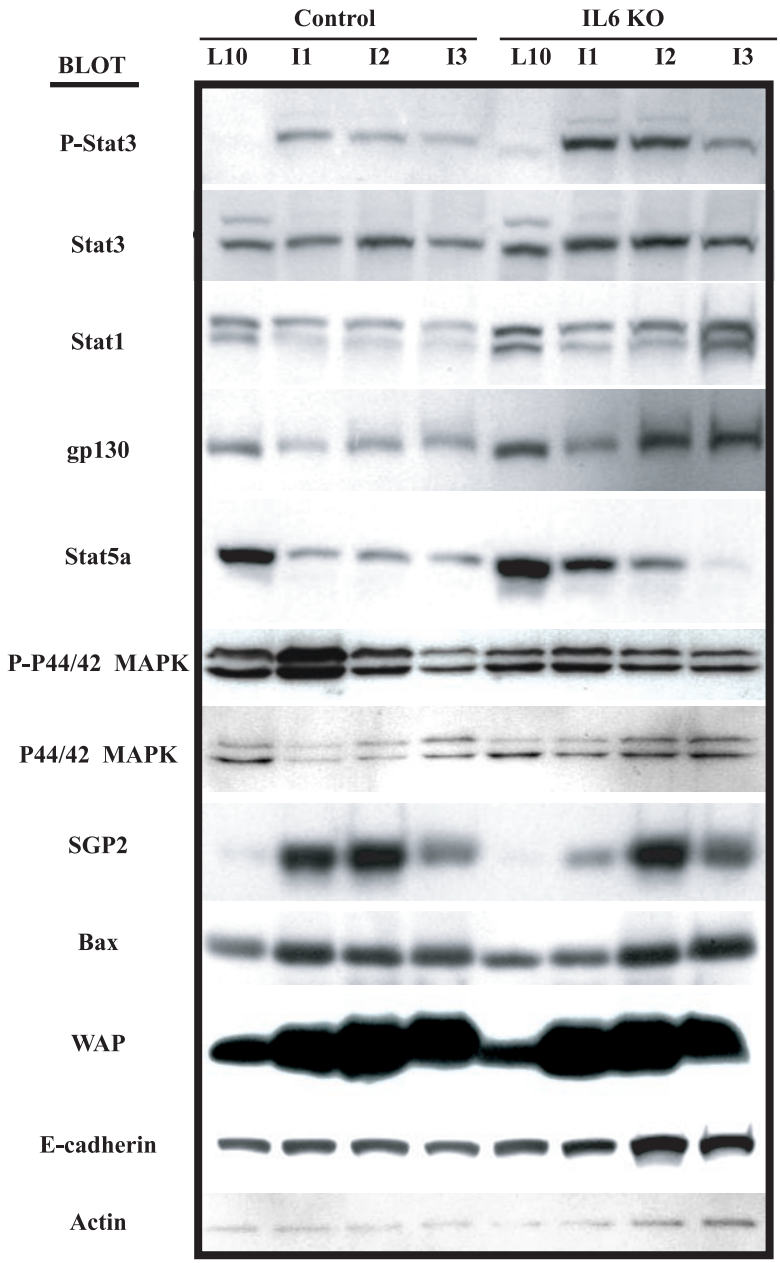

Fig. 7. Western Blot of Stat3, Stat1, gp130, Stat5a, p44/42 (ERK1/2) MAPK, SGP2, Bax, WAP, Actin, and E-Cadherin on L10, d 1, d 2, and d 3 Involution (I1, I2, I3) Mammary Glands

Actin and E-cadherin were used as loading controls. P-Stat3, Phosphorylated Stat3-Tyr (705); P-p44/42 MAPK, phosphorylated p44/42 (ERK1/2) MAPK.

T47D breast cancer cells (11). In T47D cells, IL-6 mediates growth inhibition through the activation of Stat3 and activation on MAPK-induced cell migration (11). Stimulation of proliferation in multiple myeloma cells was achieved through the activation of MAPK but not Stat3 (26). Furthermore, the disengagement of Shp-2 from gp130 signaling that affects ERK1/2 MAPK activation also resulted in a reduced cell proliferation (27). These data suggest that both Stat3 and MAPK pathways are involved in both cell proliferation and apoptosis. The differential response of target cells suggests the presence of unique downstream signaling molecules that remain to be identified.

The biological response of IL-6 may be mediated by Bcl-2 family members. P44/42 MAPK can increase Bax and p21 expression through p53 (28), and also the alteration in MAPK pathways and Bcl-2 family of proteins is involved in induction of apoptosis in rat prostate (29). The increase of Bax and Bcl-x gene expression during involution (30, 31) parallels the IL-6-induced activation of the p44/42 MAPK, suggesting that they may be genuine targets. This is supported by

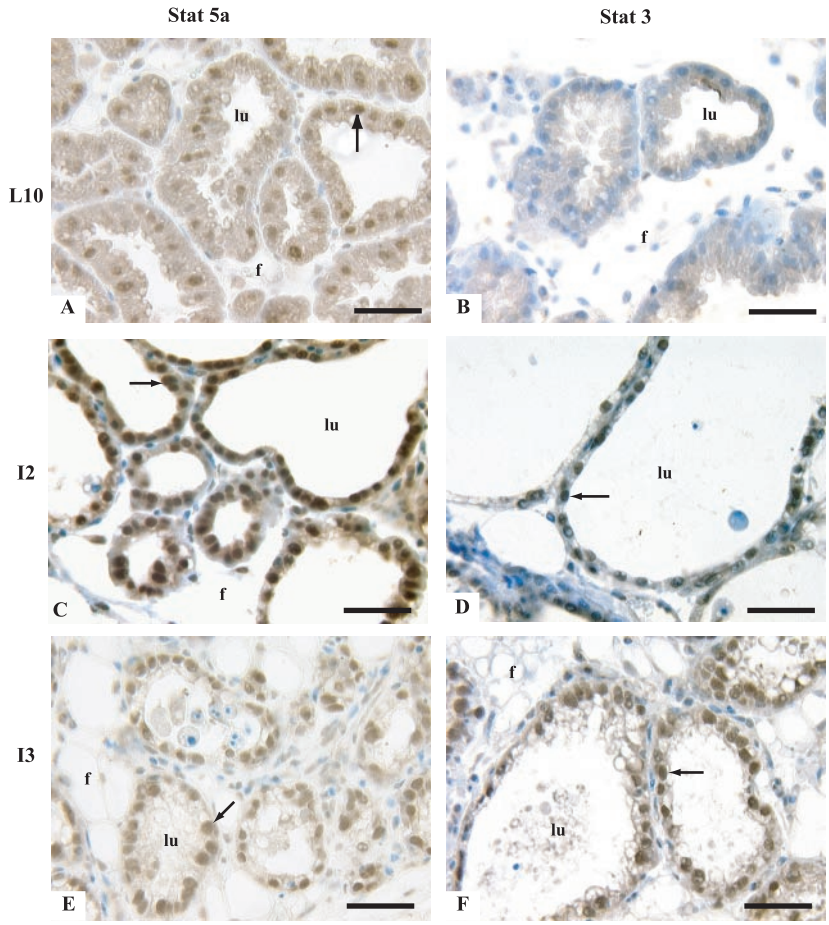

Fig. 8. Immunohistochemistry of Stat5a (A, C, and E) and Stat3 (B, D, and F) in L10 (A and B), Involution d 2 (C and D), and Involution d 3 (E and F) Mammary Glands from Wild-Type Mice

Stat5a locates in cytoplasm and nuclei at L10 (A), but mainly in nuclei during involution (C and E). Stat3 mainly stays in cytoplasm during lactation (L10); however, it translocates to nuclei during involution (D and F). *Arrows* indicate nuclear staining of Stat5a (A, C, and E) and Stat3 (D and F). lu, Lumen; f, fat. *Bar*, 50 μm.

the delay of Bax induction in the absence of IL-6. The absence of Bax also results in a retarded involution (32) reminiscent of that seen in IL-6-null mice. We therefore propose that IL-6-mediated involution is, at least in part, executed by Bax. In conclusion, loss of IL-6 leads to a delayed mammary gland involution and cell death. Surprisingly, this was not accompanied by a reduction in Stat3 activity but coincided with a failure of the MAPK pathway to be activated at the onset of involution.

MATERIALS AND METHODS

Animals

All animals used in this study were 8- to 12-wk-old female C57BL/6 mice. IL-6-null mice (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals used in the course of this study were treated within published guidelines of human animal care.

Nipple-Sealing Experiments

The two right abdominal nipples (nos. 4 and 5) were sealed at L10 using surgical gel (NEXABAND, Veterinary Products Laboratory, Phoenix, AZ). The pups remained with the dam and could still nurse from the other nipples. At 12, 24, 48, and 72 h after sealing, animals were killed and the no. 4 sealed and open mammary glands were collected for RNA, protein, and histological analyses. Five mice were used at each time point.

Involution Experiments

At L10, pups were removed from both IL-6-null and wild-type mice, and the no. 4 glands from the left side were collected by biopsy. Mice were killed at d 1, d 2, and d 3 of involution, and the right no. 4 glands were collected for RNA, protein, and histological analyses. The litter sizes were between seven and 10. Five to six mice were used at each time point.

IL-6 Injection Experiments

Three virgin control mice in diestrus, two control L10, and two IL-6-null L10 mice were anesthetized using Forane (isoflurane, Baxter Healthcare Corp. Deerfield, IL) anesthetic gas.

The left no. 4 glands were removed as control glands. Murine IL-6 (PeptroTech, Inc., Sharpsburg, MD; 200 ng/200 μl saline to virgin and 10 μg/200 μl saline to L10 mice) was injected ip, and mice were killed 15 min after the injection. The right no. 4 gland was collected and subjected to morphology and protein analysis.

Histology

Tissues were fixed in 4% paraformaldehyde at 4 C overnight (O/N), dehydrated, and embedded in paraffin. Tissue blocks were sectioned at 5 μm and stained by hematoxylin and eosin. For immunohistochemistry studies, tissue sections on poly-L-lysine-coated slides were dewaxed in xylene and rehydrated in serial ethanol concentration. All tissue sections were treated by antigen unmasking reagent (Vector Laboratories, Inc., Burlingame, CA) at boiling temperature for 10 min following manufacturer's instruction. Stat5a antibody (4) was used at a 1:600 dilution. Stat3 antibody (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was diluted at 1:200. All primary antibodies were incubated with sections O/N at 4 C. Immunoperoxidase staining was performed according to the manufacturer's protocol (Vector ABC kit, DAB substrate kit, Vector Laboratories, Inc.).

The apoptosis level in mammary epithelium during involution was determined using the Apoptag peroxidase kit (S7100, Intergen, Purchase, NY) and peroxidase staining for paraffin-embedded tissue. Slides from three mice of each group were counted for apoptotic cells. At least 1000 cells were counted from each specimen and Student's *t* test was used for statistical analysis. All data are presented as mean ± SEM.

Western Blot and Immunoprecipitation

Protein was extracted from frozen tissues (1 g/10 ml lysis buffer), homogenized in lysis buffer (10 mM Tris-HCl, pH 8.0/5 mM EDTA/50 mM NaCl/30 mM $Na_4P_2O_7$/50 mM NaF/200 μM Na_3VO_4/1% Triton X-100/1 mM phenylmethylsulfonyl fluotide/5 μg/ml aprotinin/1 μg/ml pepstatin A/2 μg/ml leupeptin) using a Polytron. The homogenate was incubated on a vertical rotator at 4 C O/N, and fat was cleared from extracts by spinning in a centrifuge (Eppendorf, Madison, WI) twice at 14,000 rpm at 4 C for 20 min. Protein from each sample (100 μg) was fractionated in 8% and 14% Tris-glycine gels (Invitrogen, San Diego, CA) and transferred onto polyvinylidene difluoride (PVDF) membrane using a Western blot apparatus from Novex (San Diego, CA). After transfer and blocking with buffer (5% BSA and 2% nonfat milk/20 mM Tris HCl, pH 7.6/137 mM NaCl) at room temperature for 1 h, the membranes were incubated with primary antibodies as listed below. Rabbit antimouse antibodies, from our laboratory: Stat5a (1:20,000), WAP (1:1000); Santa Cruz Biotechnology, Inc.: Stat3 (1:1000), gp130 (1:1000), Stat1 (1:1000), Bax (1:500), SGP2 (goat-antimouse, 1:1000); Transduction Laboratories, Inc. (Lexington, KY): P-Tyr-horseradish peroxidase (1:2500). Cell Signaling (Beverly, MA): P-Stat3-Tyr (705), P-Stat3-Ser (727), Stat3, P-p44/42 (Thr202/Tyr 204) MAPK, p44/42 MAPK, P-p38 (Thr180/Tyr182) MAPK, p38 MAPK, P-SAPK/JNK (Thr183/Tyr 185) MAPK, SAPK/JNK MAPK, P-Akt (Ser473), Akt (all in 1:1000 dilution). Monoclonal antibodies, Transduction Laboratories, Inc.: E-cadherin (1:2500). Chemicon (Temecular, CA): actin (1:4000). All first antibodies were incubated with membranes for 1 h at room temperature (RT) or at 4 C O/N. Secondary antibodies are antirabbit, antigoat, or antimouse-horseradish peroxidase antibodies (Transduction Laboratories, Inc.; 1:5000) and were incubated with membranes for 30 min at RT. Proteins were visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Arlington Heights, IL). Blots were stripped using Western stripping buffer (Alpha Diagnostic, San Antonio, TX) for 20 min at RT. For immunoprecipitation, 0.5 ml (1 mg protein) of the lysate was incubated with antibodies (4 μg of Stat3 or gp130) O/N at 4 C. Protein A-Sepharose beads (60 μl; (Sigma, St. Louis, MO) were added and incubated at 4 C for 2 h. Samples were then washed three times using lysis buffer and resuspended in 2× sample buffer (Invitrogen, Carlsbad, CA) with 2% 2-mercaptoethanol, boiled for 3 min, centrifuged briefly, and loaded to 8% Tris-glycine gel (Invitrogen). Proteins were transferred onto polyvinylidene difluoride membranes and Western Blot was performed as described above.

RT-PCR

Total RNA was extracted using the acid phenol chloroform method (33). Five micrograms of total RNA were reverse transcribed using the SuperScript preamplication system (Life Technologies, Inc., Gaithersburg, MD). Two microliters of reverse-transcribed product were used in PCR amplification of IL-6 (635 bp), IL-6-R (545 bp), gp130 (550 bp), LIF (350 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 200 bp). The primers used are as follows: IL-6, forward primer, 5′-atgaagttcctctctgcaagagac-3′; reverse primer, 5′-cactaggtttgccgagtagatctc-3′ (35 cycles). IL-6-R, forward primer, 5′-ggaaggaagcagcaggcaatg-3′; reverse primer, 5′-tgcaacgcacagtgacactatg-3′ (28 cycles). gp130, forward primer, 5′-tgtcagcaccaaggattt-3′; reverse primer, 5′-gtagctgaccatacatgaagtg-3′ (35 cycles). LIF forward primer, 5′-ggcaacctcatgaaccagatca-3′; reverse primer, 5′-gcaaagcacattgctgaggagg-3′(28 cycles). GAPDH was used as a loading control, forward primer, 5′-ctcactggcatggccttccg-3′; reverse primer, 5′-accaccctgttgctgtagcc-3′(25 cycles).

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